SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Modulation of dendritic cell functions by the cell surface autophagy regulator SLAMF5 and by mitochondrial ROS

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The Examination takes place at the Department of Immunology, Faculty of Medicine, University of Debrecen, at 11:00 a.m. on 28th of September, 2018

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 1:00 p.m. on 28th of September, 2018

1. Introduction

Dendritic cells (DCs) are major participants of the immune response, capable of recognizing a wide range of microbes and danger signals. In addition to their sensory function, they coordinate the innate and adaptive arms of the immune system to deliver efficient antimicrobial responses and to ensure that these destructive responses are used solely against pathogens, but not self-structures. During infection, DCs face several challenges that generated selective pressures for the development of effective adaptation mechanisms. For instance, reactive oxygen species generated by mitochondria (mtROS) during cellular respiration both impinge and depend on signalling pathways that determine the response of cells to disruptions in cellular homeostasis, such as infection. MtROS-dependent reversible oxidation of specific targets can alter signalling processes allowing cellular adaptation in response to changes in the intracellular and extracellular environment. Autophagy is another effective mechanism that, beyond its fundamental function in cellular homeostasis, has evolved to protect against pathogens infecting the eukaryotic cell. Beyond serving as cell-autonomous defence mechanism by removing intracellular pathogens, autophagy has been integrated into systemic immune responses by regulating innate and adaptive immunity.

ROS production and autophagy are some of the most ancient mechanisms of innate immunity, yet they are major regulators of the adaptive immune response to intracellular pathogens as well as powerful modulators of the intensity of inflammatory responses. Thus, to gain a better understanding of the inflammatory process as well as the regulatory circuits governing the immune response to intracellular pathogens we aimed to investigate the modulatory role of heightened mtROS generation as well as the identification of novel autophagy regulators in DCs.

1.1. Dendritic cells: key players in the initiation and regulation of the immune response

DCs are strategically positioned in all tissues to patrol for infection and disturbances in cellular homeostasis. When they sense molecular patterns of microbial origin or signs of tissue damage via pattern recognition receptors (PRR), they orchestrate both innate and adaptive immune responses. Human DCs comprise a heterogeneous population of hematopoietic cells, which based on their tissue localization, phenotypic and functional characteristics can be classified into two major types: conventional DCs (cDCs) and plasmacytoid DCs (pDCs).

1.1.1. PDCs display a unique expression of viral sensors ensuring an effective antiviral defence mechanism in two waves

PDCs are the major producers of type I interferons (IFN) that induce resistance to viral replication in all types of cells, providing a powerful defence mechanism against viral spreading. They also contribute to the expansion of an antiviral immune response by enhancing the activity of other

immune cells including T cells, B cells, NK cells, and cDCs. They are a rare population of circulating cells, which under steady-state conditions are absent from peripheral non-lymphoid tissues. Following activation, however, they accumulate in inflamed tissues to restrict locally viral propagation. During the early phase of viral infection pDCs circulate in blood or are localized to secondary lymphoid tissues, where they can engulf non-infectious viral particles or apoptotic bodies from infected cells. The ingested viral debris at this point is recognized via endosomal receptors, namely TLR7 and TLR9. The TLR-driven acute yet transient activation of pDCs results in direct release of large amounts of type I IFNs both into the lymph and blood. It also induces the recruitment of pDCs to the inflamed tissues and establishes their subsequent responsiveness to RIG-I ligands, whose engagement then contributes to a second wave of type I IFN production at the site of infection.

1.1.2. CDC autophagy controls the burden of infectious agents while limiting inflammatory pathologies

CDCs are key participants of the inflammatory reaction, necessary for the generation of a rapid and robust antimicrobial response and for the proper activation of the adaptive immune response. As all immune responses have the potential to convey damage to host tissues, tight control of these processes is necessary to avoid excessive inflammation and maintain homeostasis. An increasing number of studies support the view that the cellular degradative pathway of autophagy has a fundamental role in restraining potentially detrimental innate immune responses. Via autophagy, the cell sequesters its own components into *de-novo* formed double-membrane-bound vesicles; called autophagosomes to be carried to lysosomes for degradation with the aim of recycling nutrients or eliminating aberrant organelles and protein aggregates. By removing oxidized proteins and organelles as well as the cellular machineries, e.g. damaged mitochondria responsible for excessive ROS production, autophagy controls the release of multiple endogenous danger signals fuelling the inflammatory process. In this way, autophagy is involved in increasing the activation threshold of the immune response thereby preventing untimely activation under steady-state conditions. In some contexts, autophagy can also enhance immune responses. It can directly eliminate intracellular pathogens by facilitating their degradation that is presumably one of the most ancient forms of immune defence. Furthermore, the particularly high basal autophagic flux of professional antigen presenting cells redirects microbial nucleic acids from the cytosol to endosomal TLRs, and delivers cytosolic microbial antigens to MHC-II loading compartments to support the activation of $CD4^+$ T cells. Moreover, circulating monocytes recruited from the bloodstream to the sites of infection are induced to differentiate into cDCs or macrophages by a program that is also highly dependent on autophagy.

Strikingly, following stimulation of the TLR4 pathway by LPS, autophagic flux is transiently reduced in activated cDCs presumably to prevent exposure of autoreactive T cells to self-antigens in the presence of strong co-stimulatory signals. Later on, however, the autophagy machinery is reactivated in order to counteract exaggerated cytokine production thereby decreasing the magnitude and duration of inflammation.

1.2. Immune modulatory role of mtROS and SLAMF receptors

Temporal and spatial regulation of DC functions is essential to steer the immune response into the desired direction. To deploy the appropriate response to an enormous number of different environmental challenges DCs must process and integrate signals generated by many signalling pathways acting within and between the responding DC and other immune cells. Whereas some receptors or ligands or even inorganic molecules have "primary" roles in the initiation of the immune response, others have "secondary", modulatory, albeit still critical functions in the regulatory process.

1.2.1. MtROS function as signalling molecules

ROS are chemically reactive molecules that can oxidize proteins, lipids and DNA and indiscriminately damage cell constituents at high levels. When tightly controlled, however, like other post-translational modifications, reversible oxidation of specific targets can alter protein functions, thereby being capable of influencing the outcome of signal transduction pathways. The mitochondrial electron-transport chain (ETC) is a major source of cellular ROS. The orderly flow of electrons down the mitochondrial ETC to complex IV results in their final deposition into molecular oxygen. However, leakage of electrons mainly at complexes I and III of the ETC leads to partial reduction of oxygen, resulting in the formation of mitochondrial ROS (mtROS). While complex I produces ROS only into the matrix, complex III can produce ROS on both sides of the mitochondrial inner membrane. Unlike ROS produced by complex I, intermembrane space ROS, generated by complex III may act as an efficient cytosolic signalling molecule having to pass only the mitochondrial outer membrane.

Several exogenous stimuli can increase mtROS levels including exogenous ROS and pro-oxidant cytokines, such as TNF generated upon phagocyte activation. Microorganisms and their components can also enhance mtROS generation. Redox modifications can impact on cellular signalling by altering protein-protein interactions, the DNA binding activity of transcription factors, and the catalytic activity of enzymes. Interestingly, redox modifications also affect other posttranslational modifications, essential for signal transduction, for instance, phosphorylation. Redox signalling is triggered by specific stimuli that induces the production and release of redox

active molecules, and is localized to certain compartments or confined areas within a cellular compartment. An extensive body of literature has established ROS-mediated cross-regulation of PRRs; however, the putative regulatory role of mtROS on signalling induced by antiviral sensors of pDCs has not been investigated yet. Since the early phase of IFN response in pDCs is mediated by endosomal TLRs, while the late phase of IFN response can also be triggered by cytosolic RIG-I, pDCs provide an ideal model to study the impact of elevated mtROS on the antiviral signalling pathways initiated by receptors with distinct subcellular localization. Due to the limited availability of these cells from human peripheral blood, to investigate the role of mtROS in the TLR9 and RIG-I- signalling pathways driving type I IFNs secretion in pDCs, we used the GEN2.2 human pDC cell line. Our results obtained using the GEN2.2 cell line were subsequently validated in primary human pDCs isolated from the peripheral blood of healthy blood donors.

1.2.2. SLAMF receptors as regulators of autophagy

Beyond recognition of microbes via PRRs, cell-cell communications mediated by various cell surface receptors such as members of the Signaling Lymphocyte Activation Molecule Family (SLAMF) are also potent regulators of the activation and duration of the inflammatory processes. Different combinations of SLAMF receptors are expressed on the surface of hematopoietic cells acting mostly as self-ligands through homotypic interactions. Engagement of SLAMF receptors results in signalling events that modulate various phases of the innate and adaptive immune response, including the differentiation of innate and classical T cell subsets as well as the T- and B cell memory response.

Recently, SLAMF1 and SLAMF4 receptors have been shown to regulate autophagy via interaction with the Beclin-1/Vps34 autophagy macrocomplex that initiates autophagosomal membrane nucleation. SLAMF1 was found to activate the autophagic flux in human chronic lymphocytic leukemia cells by stabilization of the above mentioned complex. On the other hand, association of SLAMF4 with Beclin-1 and Vps34 inhibited autophagy in the human monocytoid THP1 cells and in mouse bone marrow-derived macrophages via reducing the lipid kinase activity of Vps34.

Despite of the presence of multiple SLAM family members, their function in cDCs has hardly been explored. Based on the known autophagy regulatory functions of SLAMF receptors in other immune cells, one possible mechanism for them to modulate cDC function is via regulation of the process of autophagy. Our focus has been on SLAMF5 that is constitutively expressed on human cDCs, thus ideally placed to modulate their functions in various phases of the immune response, including the control of immune homeostasis. We set out to determine whether this receptor would affect cDC autophagy as well as the inflammatory responses of cDCs and if so, via what molecular

mechanisms. We modelled human cDCs by differentiating primary human monocytes into moDCs *in vitro,* in the presence of GM-CSF and IL-4 for 5 days.

1.3. Aims of the study

Aim 1. Clarify how pDC-controlled antiviral responses are affected by mtROS via:

- exploring how mtROS influence type I IFN production in response to ligands that activate TLR9 or RIG-I,
- identification of molecular targets and mechanism by which mtROS may affect the TLR9 and RIG-I signal transduction pathways.

Aim 2. Assess the specific involvement of SLAMF5 receptor, a member of the SLAM family of cell surface receptors, in cDC functions by

- studying the impact of SLAMF5 on the phenotype and the functional properties of LPS/IFNγ-treated cDCs,
- examining the function of SLAMF5 in the autophagic process,
- identification of some of the underlying molecular mechanisms via examining the effect of SLAMF5 on known regulatory pathways of autophagy.

2. Materials and methods

2.1. Isolation of primary human pDCs and monocytes

Peripheral blood mononuclear cells (PBMCs) were separated from human heparinized leukocyte-enriched buffy coats by Ficoll-Paque (GE Healthcare) density gradient centrifugation. PDCs were purified by magnetic cell sorting using the human CD304 (BDCA-4/Neuropilin-1) MicroBead Kit (Miltenyi Biotech). After separation on VarioMACS magnet, pDCs were cultured in 48-well cell culture plates at a density of $5x10^5$ cells ml^{-1} in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (Life Technologies), 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin (all from Sigma-Aldrich), and 50 ng ml^{-1} recombinant human IL-3 (Peprotech).

Positive selection for monocytes from PBMCs was performed using CD14 antibody-coated magnetic microbeads (Miltenyi Biotech) according to the manufacturer's protocol.

2.2. RNA interference and moDC generation

To reduce the level of SLAMF5 in monocytes the following 25-nt Stealth™ RNAi oligonucleotides were ordered from ThermoFisher Scientific:

SLAMF5 sense:5'-UGGCUAUGUUCUUUCUGCUUGUUCU-3'

SLAMF5 antisense:5'-AGAACAAGCAGAAAGAACAUAGCCA-3'

neg. control for SLAMF5 sense:5'-UGGUAUGCUUUCUGUUCGUUUCUCU-3'

neg. control for SLAMF5 anti-sense:5'-AGAGAAACGAACAGAAAGCAUACCA-3'

IRF8-(Assay ID: s7100) and TRIM21-(Assay ID: s13462) specific Silencer Select siRNAs and non-targeted Silencer Select Negative Control No 1 siRNA were purchased from ThermoFisher Scientific. The siRNA duplexes were delivered by electroporation using GenePulser Xcell instrument (Bio-Rad Laboratories). Transfected cells were cultured at a density of 10^6 cells ml^{-1} in RPMI-1640 medium, supplemented with 10% FBS (both from ThermoFisher Scientific), 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin (both from Sigma-Aldrich), 80 ng ml^{-1} GM-CSF (Gentaur Molecular Products), and 100 ng ml^{-1} IL-4 (PeproTech) for 5 days to generate moDCs. Culture medium was refreshed on day 2 by removing three-quarters of the supernatant and replacing it by complete medium containing GM-CSF and IL-4.

2.3. Culturing of GEN2.2 cells

The human plasmacytoid dendritic cell line GEN2.2 was grown on a layer of mitomycin C (Sigma-Aldrich)-treated murine MS5 feeder cells in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (Life Technologies), 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin (both from Sigma-Aldrich) and 5% non-essential amino acids (Life Technologies).

For experiments, the GEN2.2 cells were removed from the feeder layer and seeded on 24-well plates in complete RPMI 1640 medium (Sigma-Aldrich).

2.4. Induction of elevated level of mtROS

MtROS production was monitored by loading GEN2.2 cells with 5 μM MitoSox™ Red mitochondrial superoxide indicator (Life Technologies) according to the manufacturer's recommendations. For enhanced generation of mtROS cells were conditioned with 0.5 μg ml^{-1} Antimycin-A (AMA; Sigma-Aldrich) for 6 hours. As a control, cells were also treated with MitoTEMPO (300 μM, Sigma-Aldrich), a mitochondria-targeted antioxidant 1 hour prior to and along with the AMA treatment. At the end of the designated treatments the fluorescence intensity of MitoSox™ Red was measured at 580 nm with FACS Calibur flow cytometer and data were analyzed by FlowJo software (Treestar).

2.5. Receptor cross-linking

MoDCs suspended at a density of 10^7 ml^{-1} were incubated in complete medium containing 10 μg ml^{-1} anti-SLAMF5 antibody (clone 152-1D5; LifeSpan BioSciences, Cat.No. LS-C134663) or an IgG isotype control antibody (Biolegend, Cat.No. 400124) at 4 ºC for 45 min. Cells were then washed, suspended in complete medium containing 10 μg ml^{-1} anti-$F(ab')_2$ of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Cat.No. 115-006-062), re-seeded into 24-well cell culture plates and incubated at 37 ºC for 2 hours in a humidified atmosphere containing 5% CO2.

2.6. Cell stimulation

For TLR9-mediated type I IFN production, GEN2.2 cells or primary pDCs were treated with 1 μM CpG-A (ODN 2216; Cat.No. HC4037 from Hycult Biotech) for 6 hours. To induce RIG-I receptor expression GEN2.2 cells or primary pDCs were incubated with 0,25 μM CpG-A for 16h. The cells were then washed and added back to plates in fresh medium. To achieve stimulation of RIG-I 5'ppp-dsRNA (Cat.No. tlrl-3prna from InvivoGen) was applied through Lyovec transfection reagent (InvivoGen) according to the manufacturer's recommendations. Briefly, 25 μl of the 5'ppp-dsRNA-LyoVec complex containing 1 μg ml^{-1} working concentration of the RIG-I ligand (RIGL) was added to the cells for the indicated time periods in all experiments.

MoDC maturation was induced by simultaneous addition of 100 ng ml^{-1} LPS (Ultrapure lipopolysaccharide from Salmonella minnesota R595, Cat.No. tlrl-smlps) and 10 ng ml^{-1} recombinant human IFNγ (PeproTech, Cat.No. 300-02) for the indicated time periods. For autophagy induction, immature moDCs were exposed to 50 nM rapamycin (Merck, Cat.No. 553210) for 4 hours. In some experiments cells were incubated with 20 nM bafilomycin A1

(BafA1; InvivoGen, Cat.No. tlrl-baf1) or 1 μM MG132 (SelleckChem, Cat.No. S2619) for the last 2 hours.

2.7. Western blotting

For protein extraction, cells were lysed in Laemmli buffer. Protein extracts were resolved by SDS-PAGE and transferred electrophoretically onto nitrocellulose membranes (Bio-Rad Laboratories). Non-specific binding sites were blocked with 5% non-fat dry milk or 5% BSA in case of phospho-IRF3. Membranes were probed with the following primary antibodies: anti-RIG-I (Cat.No. 4520), anti-phospho-IRF7 (Ser477; Cat.No. 12390), anti-IRF7 (Cat.No. 4920), anti-IRF3 (Cat.No. 4302), anti-MAVS (Cat.No. 3993), anti-phospho-p70S6K (Thr389; Cat.No. 9206), anti-p70S6K (Cat.No. 9202), anti-IRF8 (Cat.No. 5628S) all from Cell Signaling, anti-SLAMF5 (clone H128), anti-β-actin (Cat.No. sc-47778), anti-Akt1 (Cat.No. sc-5298), anti-ubiquitin (Cat.No. sc-9133), anti-TRIM21 (Cat.No. sc-25351) all from Santa Cruz Biotechnology, anti-EAT-2 (Cat.No. LS-C169054, LifeSpan BioSciences), anti-LC3 (Cat.No. NB100-2220, Novus Biologicals), anti-phospho-Akt (Ser473; Cat.No. AF887, R&D System) or anti-phospho-IRF3 (S386; Cat.No. ab76493, Abcam). Bound primary antibodies were detected with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (GE Healthcare). Signals were developed by using SuperSignal West Pico or Femto chemiluminescent substrates (Thermo Scientific) and film exposure. Densitometric analysis of immunoreactive bands was performed using the Kodak 1D Image Analysis Software version 3.6. To ensure equal protein loading β-actin served as loading control while the level of phosphorylation was normalized to the total amount of the same protein present in the samples.

2.8. RNA extraction, reverse transcription and real-time quantitative PCR

Extraction of total RNA was performed using TRI-Reagent (Molecular Research Center) according to the protocol of the manufacturer. 1 μg of total RNA was treated with DNase I (Thermo Scientific) to exclude amplification of genomic DNA, then reverse transcribed into cDNA using the High Capacity cDNA RT Kit of Applied Biosystems. The cDNA product was used for real time qPCR reactions using Dream Taq DNA Polymerase (Thermo Scientific) and the following gene-specific primers according to the manufacturer's instructions: IFNA1 (Assay ID Hs.PT.49a.3184790.g), cyclophilin from Integrated DNA Technologies and IRF8 (TermoFisher Scientific, Assay ID: Hs00175238_m1). Quantitative PCR was performed using the ABI StepOne Real-Time PCR System (Applied Biosystems) and cycle threshold (CT) values were determined using the StepOne v2.1 Software (Applied Biosystems). The relative amount of mRNA ($2^{-\Delta CT}$) was obtained by normalizing to the cyclophilin house keeping gene in all experiments.

2.9. Flow cytometry

Cell viability was determined by 7-aminoactinomycin-D (7-AAD; 10 μg ml^{-1}; Sigma-Aldrich) staining for 15 min immediately before flow cytometric analysis. Cell surface protein expression was analysed with FITC-labelled monoclonal antibodies against HLA-A, B, C (Sony Biotechnology), HLA-DQ, CD40 (all from BioLegend), PE-tagged monoclonal antibodies against SLAMF5 (BioLegend, clone 1.21), CD14, CD86 (both from R&D Systems), DC-SIGN (Sony Biotechnology) and APC-conjugated monoclonal antibodies against CD1a (BioLegend). Isotype-matched control antibodies were obtained from BioLegend. Measurement of autophagy was performed using the Cyto-ID Autophagy detection kit (Enzo Life Sciences). Cells were incubated with Cyto-ID dye (1:2000) for 30 min at 37 C, then were washed and immediately subjected to flow cytometry. Fluorescence intensities were measured with a FACS Calibur cytometer and data were analysed with the FlowJo software (TreeStar).

2.10. ELISA

The concentration of the secreted IL-1β and IL-12 was determined by the BD-OptEIA Human ELISA kits (BD Biosciences). The level of IL-23 was evaluated by the human IL-23 ELISA Ready-Set Go kit (eBioscience). The pre-coated human IFNα ELISA kit was purchased from PBL InterferonSource. Assays were performed according to the manufacturer's instructions. Absorbance measurements were carried out by a Synergy HT microplate reader (Bio Tek Instruments) at 450 nm.

2.11. Statistical analysis

Data are expressed as mean ± SD. All results were confirmed in at least three independent experiments. Data were analysed with GraphPad Prism v.6. software. Statistical differences among the experimental groups were determined by Student's unpaired *t* test or ANOVA, followed by Bonferroni *post hoc* analyses for least-significant differences. P-values of <0.05 were considered to be statistically significant.

3. Results

3.1. Regulation of type I interferon responses by mtROS in pDCs

3.1.1. Raising mtROS level in GEN2.2 cells by the complex III inhibitor AMA

Studies aiming at understanding pDC-controlled antiviral responses have been hampered by the paucity of these cells in peripheral blood. To overcome this limitation we used the human pDC cell line GEN2.2, widely accepted by the field as a reliable surrogate for pDCs. Based on previous publications, ROS play an essential role in the regulation of the virally-stimulated signalling pathways. To evaluate how changes in the redox state induced by mitochondria influence activation of specific PRRs in pDCs, we treated GEN2.2 cells with Antimycin-A (AMA). AMA is a well-known complex III inhibitor that enhances the release of ROS into the mitochondrial matrix and to the intermembrane space. By the use of the fluorescent dye, MitoSox^{TM} that selectively detects superoxide in the mitochondria of live cells, and the mitochondria-targeted antioxidant, MitoTEMPO, we evaluated that 0,5 μg ml^{-1} AMA is the optimal concentration for inducing accumulation of ROS in GEN2.2 cells.

From these experiments we could conclude that AMA treatment of GEN2.2 cells is suitable as an *in vitro* model of the *in vivo* stress conditions and/or metabolic changes induced by viral infection.

3.1.2. MtROS inhibit CpG-A-induced type I IFN production in GEN2.2 cells by reducing the phosphorylation level of IRF7

First, we evaluated the possible consequences of increased mtROS generation in pDCs on the initial wave of IFNα production induced via TLR9. We found significantly up-regulated IFNA1 mRNA production and IFNα secretion upon exposure to the TLR9 ligand CpG-A, which effect was abrogated when cells were activated in the presence of elevated levels of intracellular ROS triggered by treatment with AMA. In the attempt to understand how mtROS may affect the TLR9 signalling pathway, we analysed the impact of mtROS on phosphorylation of IRF7, known to play a critical role in TLR-induced type I IFN gene transcription in pDCs. These experiments revealed significantly attenuated CpG-A-induced IRF7 phosphorylation in the presence of AMA.

Taken together, these data show that excess ROS induction in the mitochondria blocks CpG-A-induced transcription of IFNA1 mRNA and hence IFNα secretion of GEN2.2 cells, presumably via inhibition of IRF7 phosphorylation by mtROS.

3.1.3. Elevated level of mtROS abrogates CpG-A-induced RIG-I expression in GEN2.2 cells

A key molecular feature of pDCs is a very low basal expression of RIG-I under steady-state conditions. RIG-I, however, is rapidly and dramatically upregulated upon stimulation by endosomal

TLR ligands. Based on this previous observation, to address the effect of CpG-A on RIG-I expression in GEN2.2 cells, we first determined by titration that exposure of GEN2.2 cells to 0,25 μM CpG-A for 16 hours is the optimal condition to induce RIG-I expression. Our experiments revealed that AMA-treatment restricts the capacity of CpG-A to increase RIG-I protein level.
Altogether, these data point to the essential negative regulatory role of mtROS both in the early wave of type I IFN responses and in the endosomal TLR-induced expression of RIG-I.

3.1.4. MtROS synergize with RIG-I ligand to stimulate type I IFN production in GEN2.2 cells

Although elevated level of mtROS seems to be a strong inhibitory signal for the induction of RIG-I expression, we have no information about the effect of mtROS on RIG-I signalling, once RIG-I had been induced prior to the release of mtROS. Thus, we investigated the effects of mtROS on RIG-I-induced IFNα production. As observed previously in primary pDCs, RIGL treatment stimulated IFNA1 gene transcription and IFNα protein secretion in GEN2.2 cells as well. Remarkably, an even greater enhancement of IFNA1 mRNA and IFNα protein production was observed when cells were activated by RIGL in the presence of AMA. These data suggested a context-dependent regulatory role for mtROS on type I IFN production. Thus, we investigated whether the opposing regulatory effects of elevated mtROS on TLR9- and RIG-I-mediated cellular responses depend on the PRR or the activation state of the cell at the time of mtROS induction. To this end, cells were pre-treated with CpG-A (0,25 μM) as described previously, then, instead of RIGL, they were re-activated with a higher dose of CpG-A (1 μM) in the presence or absence of AMA. Elevated level of mtROS inhibited the type I IFN production induced by CpG-A re-stimulation demonstrating that the regulatory effects of mtROS was PRR specific.

3.1.5. MtROS affect type I IFN production of primary pDCs in a PRR-dependent manner

Thus far, we used a human transformed pDC line. To examine whether the phenotype observed with GEN2.2 cells translates to primary pDCs, we isolated primary human pDCs from peripheral blood of healthy donors and activated the cells as described for GEN2.2 cells. These experiments showed that, consistent with our observation made with the GEN2.2 cell line, type I IFN production initiated by CpG-A was abolished by mtROS in primary pDCs, whereas it was significantly enhanced in response to activation with RIGL.
These results validate the opposing, context-dependent modulatory role of mtROS in the TLR9- and RIG-I-mediated type I IFN production by human pDCs.

3.1.6. MtROS act as an enhancer for the key signalling molecules of the RIG-I pathway

Based on the observation that elevated mtROS increases the ability of pDCs to mount an efficient RIG-I-mediated antiviral response, the regulation of signalling molecules by mtROS downstream of RIG-I was investigated. IRF3 is crucial to drive type I IFN production in pDCs in response to RIG-I-activating ligands. We observed that RIGL-induced phosphorylation of IRF3 was augmented by concomitant treatment with AMA, which mirrors the capacity of these cells to secrete IFNα. In line with previous reports, we observed redox-dependent enhancement of MAVS expression and Akt phosphorylation, which molecules participate in RIG-I-mediated immune signalling leading to IRF3 activation and type I IFN production.
These findings raise the possibility that mtROS enhance RIG-I-induced type I IFN production via their direct, activating effect on the components of the RIG-I signalling pathway.

Taken together, data presented above demonstrate that mtROS possess a context-dependent regulatory function in both the early and the late phases of type I IFN responses of pDCs, depending on the actual viral sensing pathway stimulated. Thus, our results reveal a novel, versatile role for mtROS in the coordination of the antiviral response of human pDCs.

3.2. SLAMF5 enhances autophagy and fine-tunes cytokine response in moDCs via stabilization of IRF8

3.2.1. SLAMF5 is up-regulated during the differentiation and activation of moDCs, but is not required for their survival or phenotypic maturation

Regarding surface proteins with immune modulatory functions a number of alterations are observed during moDC differentiation. Our data show that SLAMF5 is strongly up-regulated during *in vitro* differentiation of human monocytes into immature moDCs implicating SLAMF5 as a regulator of the differentiation process. To assess the function of SLAMF5 in moDCs we performed RNA interference targeting SLAMF5 on freshly isolated monocytes, which reduced the expression of SLAMF5 by 80-95% without having a significant effect on the viability or the overall number of moDCs differentiated from monocytes. We found that, cell surface expression of CD14, highly expressed on monocytes but downregulated on moDCs, as well as DC-SIGN, typically expressed by differentiated moDCs, was identical in SLAMF5-silenced and control moDCs indicating that SLAMF5-silenced cells retained their ability to differentiate into moDCs. As simultaneous LPS and IFNγ challenge further increases SLAMF5 expression in moDCs, we decided to analyse the potential role of this receptor as a modulator of moDC activation in response to LPS/IFNγ. As judged by the expression of MHC-I, MHC-II, CD40 and CD86 expression, the phenotypic maturation of SLAMF5 knockdown moDCs is similar to control cells.

3.2.2. Manipulation of SLAMF5 signalling changes the intensity of moDC autophagy

Recent publications revealed SLAMF receptors as regulators of the autophagic process in specific immune cells. Therefore, we set out to evaluate the impact of SLAMF5 on moDC autophagy. The most widely used assay to monitor this process is measuring lipid conjugation of LC3 by western blotting as detected by changes in LC3-I and LC3-II levels. To monitor autophagosome formation, we inhibited LC3-II degradation by treating the cells with bafilomycin A1 (BafA1) that blocks autophagosome-lysosome fusion. We observed a lower LC3-II/β-actin ratio in SLAMF5-silenced moDCs compared to controls both under steady-state conditions and following activation with LPS/IFNγ. The above experiment was performed in the absence of BafA1 as well to ascertain whether SLAMF5 silencing causes blockage of autophagosome-lysosome fusion and lysosomal clearance. LC3-II levels were again lower in SLAMF5-silenced moDCs suggesting that LC3-II was readily processed by the autolysosomes. This result proved that instead of being involved in autophagosome maturation, SLAMF5 enhances autophagosome biogenesis. Flow cytometric analysis of cells loaded with Cyto-ID, a fluorescent probe that selectively labels autophagic vacuoles in live cells, validated the impact of SLAMF5 on autophagy. Consistent with these findings, cross-linking of SLAMF5 with an agonistic antibody (152.1D5) increased LC3-II levels both in the presence and absence of BafA1.

By these experiments we identified SLAMF5 as a cell-surface expressed regulator protein required for the maintenance of basal autophagy as well as the recovery of autophagy after LPS/IFNγ stimulation.

3.2.3. SLAMF5 regulates autophagy by a mechanism independent of mTOR

We examined whether SLAMF5 exerts its function on autophagy via interfering with the signalling of mTOR, the key negative regulator of autophagy. If the reduced autophagy flux in the absence of SLAMF5 is the consequence of increased mTOR activity, blocking mTOR is expected to reverse the effect of SLAMF5 silencing. Our observation that the mTOR inhibitor rapamycin did not modify the extent of autophagy defect elicited by SLAMF5 silencing made unlikely that SLAMF5 modulates autophagy through modulation of the mTOR pathway. To further test this hypothesis, we examined the phosphorylation of Akt, a protein activated upstream of mTOR as well as the mTOR substrate p70S6K in response to LPS/IFNγ within a 4-hour time period. We observed that the phosphorylation of Akt and p70S6K was unaffected by SLAMF5 depletion.

These results together suggest that SLAMF5 regulates autophagy independently of mTOR.

3.2.4. SLAMF5 enhances autophagy by blocking TRIM21-dependent proteasomal degradation of IRF8

In search of the molecular mechanism by which SLAMF5 promotes autophagy we next focused on the IRF8 transcription factor on the basis of its central role in autophagy. Studies on murine bone-marrow derived DCs show that under steady state conditions IRF8 is expressed at low levels while in response to LPS/IFNγ its expression is strongly stimulated, which in turn activates many genes involved in all phases of autophagy. First, we tested the autophagy regulatory effect of IRF8 in human moDCs by siRNA-mediated gene silencing. We found that human DCs, like their murine counterparts, use IRF8 for the regulation of autophagy. Next, we presented evidence that identified IRF8 as a downstream element of the SLAMF5 regulatory pathway by demonstrating that the increased autophagy, induced by SLAMF5-cross-linking, was dependent on the presence of IRF8. Our experiments on SLAMF5-silenced moDCs revealed greatly reduced amount of IRF8 protein levels, but no difference in the amount of IRF8 mRNA. Activating the cells in the presence of the proteasome inhibitor MG132 restored the availability of IRF8 in SLAMF5 knockdown moDCs, indicating that SLAMF5 affects the proteasomal degradation of IRF8. In line with previous findings that the TRIM21 E3 ubiquitin ligase catalyses the ubiquitination of IRF8 in murine macrophages, leading to its proteasomal destruction, we observed that IRF8 degradation provoked by SLAMF5 depletion depends on the presence of TRIM21. Finally, we excluded the participation of EAT-2, the only known adapter protein of SLAMF receptors in APCs, in transmitting SLAMF5 signals by demonstrating its absence both in resting and LPS/IFNγ-activated moDCs.

According to these results, although SLAMF5-silenced moDCs are capable of up-regulating IRF8 synthesis, have nevertheless decreased IRF8 protein level due to the increased proteasomal degradation of this protein. This process appears to be dependent on TRIM21 but independent of the SLAM family-specific adaptor EAT-2. Based on this, a possible mechanism connecting SLAMF5 to autophagy relies on sustained activity of IRF8, a major transcription factor of autophagy-related genes.

3.2.5. SLAMF5 and IRF8 silencing in monocytes results in development of moDCs with overlapping changes in phenotype and cytokine secretion

To strengthen our assumption that the impact of SLAMF5 silencing on moDC functions is the consequence of IRF8 degradation, hereinafter, we investigated whether loss of IRF8 replicates the changes in moDC phenotype and cytokine production, which can be provoked by SLAMF5 silencing. A previous study by Granato et al. reported that interference with the autophagic process in monocytes resulted in reduced expression of CD1a on moDCs. We found that, silencing either

SLAMF5 or IRF8 in monocytes significantly decreased the percentage of $CD1a^+$ moDCs, consistent with the state of reduced autophagy.

One of the major roles of autophagy in cDCs is to set a limit to the production of pro-inflammatory cytokines, thus when autophagy is defective the inflammatory process is not properly controlled. In line with this, defective autophagy in SLAMF5- and IRF8-silenced moDCs coincides with enhanced secretion of IL-1β and IL-23. However, IL-12 secretion was lower in both types of knockdown cells reflecting the IRF8-dependent production of this cytokine.

The finding that depletion of IRF8 results in similar phenotypic and functional changes to those seen in SLAMF5 knockdown moDCs, further supports that IRF8 is part of the SLAMF5 autophagy regulatory pathway.

In summary, our work reveals a novel link between the SLAMF- and IRF8-regulated pathways and establishes SLAMF5 as a cell surface-expressed regulator of moDC autophagy that fine-tunes cytokine production of human moDCs.

4. Discussion

Several molecular cues guide DC functions obtained through multiple cross-talk mechanisms between DCs and other cells of their local microenvironment and also via intrinsic cellular mechanisms such as signalling from their own mitochondria. In our work, we first examined how pDC functionality is affected by mitochondrially produced ROS. In parallel, we investigated the regulatory role of cell-cell communication by studying the modulatory effects of the cell surface receptor SLAMF5 on the autophagic process of cDC and exploring the mechanism behind it.

The dynamic balance between the production and elimination of ROS is disturbed when the host becomes infected with a virus. Increased ROS might modulate infected cell responses, immune defences, viral replication and contribute to the pathogenesis of infections. Our group has previously shown that TLR7-induced cellular responses of primary human pDCs are highly sensitive to oxidative stress. Thus, in inflamed peripheral tissues, where pDCs are exposed to ROS, TLR-mediated pathways are presumably suppressed. Research described in this work extended our current understanding of pDC functions significantly by showing that similar to exogenous H_2O_2, mtROS restrain endosomal TLR-induced production of IFNα, irrespective of the activation state of pDCs. By contrast, we found that mtROS amplify RIG-I signalling in human pDCs. We also demonstrated that key components of the RIG-I pathway, such as the IRF3 transcription factor, the adaptor molecule MAVS and the Akt protein kinase are positively regulated by mtROS. Based on these observations, we propose that during the early phase of viral infection, systemic type I IFN response of pDCs is driven by TLR-mediated signals, while their late, local antiviral response - when pDCs gain access to peripheral tissues - might be mediated primarily by the cytosolic receptor, RIG-I.

These findings, however, raise the question of how this context-dependent regulation of type I IFNs by mtROS is to the advantage of the host? We propose that in inflamed tissues, elevated level of ROS contributes to diminish the amplitude of type I IFN signalling via TLRs, probably to avoid destruction of healthy tissues. It is not surprising that several viruses developed strategies to increase mtROS, thus might usurp the above regulatory mechanism to escape TLR-induced antiviral responses unleashed by pDCs. Perhaps the most significant discovery of the above described findings is the identification of the RIG-I-dependent “salvage pathway” for type I IFN production by which under virally-induced oxidative stress the RIG-I pathway could compensate for the lack of a functional TLR pathway and support immunity to viruses. Therefore, the TLR-induced RIG-I signalling pathway in pDCs might be one of the crucial mechanisms to circumvent virus escape from the innate immune response. Throughout evolution a wide array of tactical solutions has emerged by which viruses could evade host innate defences that continuously forced the host’s immune system to evolve mechanisms that overcome viral infections. It is tempting to

speculate that the crosstalk of TLR and RIG-I signalling cascades in pDCs and their opposing regulation by mtROS are the consequence of another battle of the ancient war between virus and host.

Another mechanism to ensure protective antimicrobial responses while reducing the severity of inflammation and tissue damage is via the strict regulation of autophagy in cDCs. This requires communication between sensors of the immune environment and the regulatory pathways of autophagy. Our experiments identified SLAMF5 as a novel cell surface regulator of cDC autophagy that operate via IRF8, a transcription factor of many autophagy genes. We observed a TRIM21-dependent degradation of IRF8 in SLAMF5-silenced cells, suggesting that SLAMF5 sustains IRF8 signalling in cDCs via inhibiting this E3 ubiquitin ligase.

In human PBMCs a consequence of autophagic block, either by the PI3K inhibitor 3-methyladenine or by knockdown of the autophagy protein Atg7, is increased production of IL-1β in response to either LPS or Mycobacterium tuberculosis (Mtb). Likewise, inhibition of autophagy in moDCs allowed excessive IL-23 secretion, whereas induction of autophagy had the opposite effect. We found that SLAMF5- and IRF8-silenced moDCs stimulated with LPS/IFNγ showed a similar increase of IL-1β and IL-23 production as autophagy-deficient cells. However, impaired autophagy in both SLAMF5- and IRF8-silenced cells was strikingly accompanied by decreased IL-12 secretion. This seems to be in contradiction with previous studies describing increased IL-12 production in case of autophagic block. This was demonstrated by both myeloid cell-specific deletion of the autophagy protein Atg5 in mice followed by Mtb infection, and treating microglia with the PI3K inhibitor 3-methyladenine as well as silencing Atg5 or Beclin-1 by RNA interference prior to LPS stimulation. This discrepancy, though, may be readily resolved by considering the IRF8 dependence of LPS/IFNγ-induced IL-12 production in macrophages and, as our results demonstrate, in human moDCs.

Beyond suppressing inflammation, autophagy partakes in direct elimination of pathogens, when they manage to invade the host cell interior. For this reason, many intracellular pathogens have evolved strategies to interfere with the autophagic microbicidal defence. It was recently reported that hepatitis C virus (HCV), derived from sera of infected patients, blocked the autophagic process in differentiating monocytes, which resulted in impaired transition of $CD1a^-$ cells to $CD1a^+$ moDCs. The authors speculated that interfering with moDC development via block of autophagy is part of the escape strategy by which HCV may keep the antiviral response under control. Importantly, we observed a similar decrease in the subset of $CD1a^+$ moDCs in the absence of both SLAMF5 and IRF8.

In contrast with the ubiquitous basal autophagy apparatus, SLAMF5 is expressed solely on hematopoietic cells. Thus, SLAMF5 is likely part of a regulatory module required for the fine-

tuning of autophagy in specific immune cells. However, whether its autophagy regulatory effect varies in different immune cell types still needs to be established. Considering the protective role of autophagy during an antimicrobial immune response, enhancing autophagy in immune cells may be beneficial in treatments of infectious diseases.

Altogether, our experiments identified SLAMF5 as a novel cell surface modulator of autophagy and revealed an unexpected link between the SLAMF and IRF8 signalling pathways, both implicated in multiple human pathologies. Additional work is required to establish how this novel pathway may be harnessed to modulate regulatory circuits of autophagy and inflammation to improve current therapies in various infectious and/or autoimmune diseases.

5. Summary

Inflammation today is considered as an essential, integral part of the immune response. Uncontrolled, exuberant or chronic inflammation however leads to extensive tissue damage that can contribute to the development of multiple human pathologies including various autoimmune diseases and cancers. The two major subsets of dendritic cells (DC), the conventional (cDC) and the plasmacytoid DCs (pDC) play an instrumental role in setting the threshold and regulating the length and intensity of the inflammatory response. The common theme in this work was to study the role of extracellular and intrinsic signals on the antimicrobial mechanisms of DCs against intracellular pathogens. More specifically, we analysed the role of mitochondrial reactive oxidative species (mtROS) on the activity of TLR- and RIG-I-induced signalling in pDCs and studied the role of SLAMF5, a member of the Signaling Lymphocyte Activation Molecule Family (SLAMF) on the regulation of autophagy in cDCs.

Generation of mtROS, triggered by stress or metabolic changes in the cells, is one of the key regulators of virus-stimulated signalling pathways. We found that in TLR9 agonist-induced pDCs elevated level of mtROS markedly reduced the expression of type I interferon (IFN) genes via blocking phosphorylation of IRF7, the key transcription factor of type I IFNs. In contrast, mtROS enhanced the expression of type I IFN genes induced by RIG-I agonist in pDCs via increasing the expression and phosphorylation of stimulatory signalling proteins in this pathway. The identified novel mechanism allows pDCs to maintain viral sensing and activation in the inflammatory environment, where TLR9-induced signalling is inhibited by the elevated levels of ROS.

SLAMF receptors are cell surface proteins, expressed on hematopoietic cells, which facilitate communication among immune cells. This work describes the identification of SLAMF5 as a new member of cell-surface expressed regulators of autophagy. We discovered that, unlike SLAMF1 and SLAMF4 that are regulators of the Vps34/Beclin-1 autophagy macrocomplex, SLAMF5 exerts its regulatory function via inhibiting proteolytic degradation of IRF8, a master regulator of autophagy by a mechanism dependent on the E3 ubiquitin ligase TRIM21. As an autophagy regulator, SLAMF5 influences the ratio of $CD1a^{+}$ cells in differentiating cDCs and partakes in the regulation of IL-1β, IL-23 and IL-12 production in LPS/IFNγ-activated cDCs in a manner that is consistent with its effect on IRF8 stability.

Considering the well-established role of ROS and autophagy in chronic inflammation, autoimmune diseases and cancer, the novel mechanisms presented in this work should significantly contribute to a better understanding and perhaps management of these debilitating or fatal diseases.

6. Publications

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Candidate: Zsófia Agod
Neptun ID: IWCMYY
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. Agod, Z., Pázmándi, K. L., Bencze, D., Vereb, G., Bíró, T., Szabó, A., Rajnavölgyi, É., Bácsi, A., Engel, P., Lányi, Á.: Signaling Lymphocyte Activation Molecule Family 5 Enhances Autophagy and Fine-Tunes Cytokine Response in Monocyte-Derived Dendritic Cells via Stabilization of Interferon Regulatory Factor 8.
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2. Agod, Z., Fekete, T., Budai, M. M., Varga, A., Szabó, A., Moon, H., Boldogh, I., Bíró, T., Lányi, Á., Bácsi, A., Pázmándi, K. L.: Regulation of type I interferon responses by mitochondria-derived reactive oxygen species in plasmacytoid dendritic cells.
   Redox Biol. 13, 633-645, 2017.
   DOI: http://dx.doi.org/10.1016/j.redox.2017.07.016.
   IF: 6.337 (2016)

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List of other publications

3. Pázmándi, K. L., Agod, Z., Kumar, B. V., Szabó, A., Fekete, T., Somogyi, V., Veres, Á., Boldogh, I., Rajnavölgyi, É., Lányi, Á., Bácsi, A.: Oxidative modification enhances the immunostimulatory effects of extracellular mitochondrial DNA on plasmacytoid dendritic cells. Free Radic. Biol. Med. 77, 281-290, 2014.
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4. Szabó, A., Osman, R. M., Bacskai, I., Kumar, B. V., Agod, Z., Lányi, Á., Gogolák, P., Rajnavölgyi, É.: Temporally designed treatment of melanoma cells by ATRA and polyI:C results in enhanced chemokine and IFNb secretion controlled differently by TLR3 and MDA5. Melanoma Res. 22 (5), 351-361, 2012.
DOI: http://dx.doi.org/10.1097/CMR.0b013e328357076c
IF: 2.518

Total IF of journals (all publications): 21,02
Total IF of journals (publications related to the dissertation): 12,766

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár

List of presentations

Agod Zs, Lányi Á: Characterization of CD84 splice variants in immunocompetent cells. 39th Congress of the Hungarian Immunological Society, 4-7. November 2010, Szeged, Hungary

Agod Zs, Lányi Á: The role of SLAM receptors in the regulation of dendritic cell functions. 5th Molecular Cell and Immune Biology Winter Symposium, 4-7. January 2012, Galyatető, Hungary

Agod Zs. Lányi Á: Differential regulation of TLR- and CD40-induced dendritic cell functions by SLAM-family receptors. 26th Annual European Macrophage & Dendritic Cell Society Meeting, August 31-September 2. 2012, Debrecen, Hungary

Agod Zs, Lányi Á: The role of SLAMF1 and SLAMF5 in the CD40L- and TLRL-induced dendritic cell functions. 41th Congress of The Hungarian Immunological Society, 17-19. October 2012, Debrecen, Hungary

Agod Zs, Lányi Á: SLAMF5 is a regulator of CD40L-induced dendritic cell responses. 8th ENII Immunology Summer School, May 27 - June 03. 2013, Alghero, Italy

Agod Zs, Lányi Á: SLAMF5 is a regulator of CD40L-induced responses in plasmacytoid dendritic cells. 7th Molecular Cell and Immune Biology Winter Symposium, 7-10. January 2014, Galyatető, Hungary

Agod Zs, Lányi Á: SLAMF5 regulates the inflammatory character of plasmacytoid dendritic cells.13th International Symposium on Dendritic Cells, 14-18 September 2014, Tours, France

Agod Zs, Lányi Á: DC autophagy in response to LPS and IFNγ is controlled by SLAMF5 via p38 and IRF8. 30th Annual European Macrophage & Dendritic Cell Society Meeting, 21-23. September 2016, Amsterdam, Netherlands

Agod Zs, Lányi Á: SLAMF5, a novel positive regulator of DC autophagy. 46th Congress of the Hungarian Immunological Society, 18-20. October 2017, Velence, Hungary

7. Keywords

dendritic cell, mitochondrial ROS, SLAMF5, Toll-like receptor, RIG-I receptor, interferon, autophagy, antiviral response, inflammation

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